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BREATH ANALYSIS FOR MEDICAL DIAGNOSIS

Complex mixtures of volatiles in breath form 'fingerprints' that can be used to detect disease

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TAKE A DEEP BREATH AND EXHALE. Oxygen in and CO_2 out. It's an easy-to-remember, straightforward bit of science that grade school kids know. The trouble is, the simple description of gas exchange from breathing ignores the enormous number of volatile organic compounds that come and go through our bodies with every breath.

The rich assortment of chemical substances present in an individual's breath can reveal a great deal about the person doing the breathing. Researchers have been tapping into that data pool to develop breath analysis techniques that form the basis of noninvasive and painless medical testing procedures for early detection of some diseases and other medical conditions. A number of scientists active in the field gathered at a Pittcon symposium to report on and learn about the latest advances in breath analysis.

Kicking off the session, Michael Phillips presented a brief history of breath analysis and discussed some of the technical hurdles that need to be surmounted to develop reliable medical tests. Described by his colleagues as a pioneer in diagnostic breath analysis, Phillips is the founder and chief executive officer of Menssana Research, a Newark, N.J.-based company that has developed breath tests for detecting heart transplant rejection, lung and breast cancer, and other diseases. He is also a practicing physician and professor of clinical medicine at New York Medical College, Valhalla, N.Y.

Describing the physiological basis for the ready exchange of gases between ambient air and blood, Phillips showed attendees a micrograph of lung tissue. Gas exchange occurs at the surface of numerous tiny chambers known as alveoli that are present at the tips of bronchial air passages, he explained. Alveoli are well adapted to their function in that they are lined with a very thin membrane that's loaded with capillaries.

"That means that there's a tiny distance between a red blood cell moving through a capillary and the air inside an alveolus," Phillips pointed out. He explained further that the large surface area (on the order of the size of a tennis court) and tiny distances associated with alveoli afford "a ready opportunity for volatile organic compounds to diffuse from the air into the blood and vice versa."

Some components of breath are easily detected without analytical instruments, Phillips noted. Since the days of Hippocrates and the ancient Greeks, physicians have taught their students to use their sense of smell to learn about their patients' conditions. Phillips instructs his students to do the same today—and for good reason. "You can learn a lot just by smelling your patients with the unaided nose," he asserted.

For example, patients with uncontrolled diabetes often smell like rotten apples, mainly because of the acetone in their breath, he noted. People suffering from renal failure are distinguished by a urine-like odor in their breath caused by trimethylamine, and patients with lung abscesses often have a sewerlike smell, Phillips explained, for the same reason that sewers smell as they do—because of the proliferation of anaerobic bacteria.

By the 18th century, scientific methods were being used to analyze breath. In 1784, Antoine Lavoisier constructed an apparatus used to establish the presence of CO_2 in exhaled air. Other advances led to detection of ethanol and acetone in the 19th century, and in the 1970s, Linus Pauling used microconcentration techniques to detect a large number of volatile organic compounds in breath.

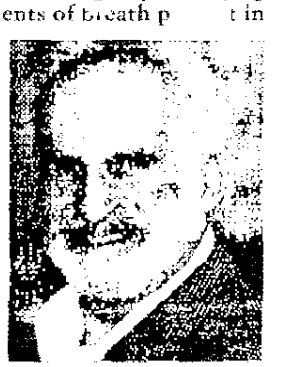
A key challenge in analyzing breath is separating the "alveolar breath" (the analyte-rich air delivered from deep in the lungs) from the "dead space" air (the volume of breath contained in the upper airways, mouth, and pharynx that is uninvolved in gas exchange). Another challenge is separating and identifying the volatile breath components, which, according to Phillips, tend to be present at just picomolar-level concentrations.

To collect samples, Phillips and coworkers use a portable sorbent-based system through which a test subject breathes for a fixed period. The device separates dead-space air (the first portion of exhaled breath) from alveolar breath and traps the organic molecules on purified samples of activated carbon. The setup is also used to collect samples of room air for background correction and features small traps that can be sealed and transported for laboratory analysis. The components are separated using standard thermal-desorption methods coupled with gas chromatography and detected and quantified via mass spectrometry.

TO DETERMINE the types and concentrations of substances that might serve as markers (indicators) for particular diseases, researchers need to begin by identifying the components of breath present in healthy individuals. In a study of that type conducted a few years ago, Phillips and coworkers analyzed breath samples from 50 people and observed some 200 volatile organic compounds per person—but with wide variation. In total, nearly 3,500 analytes were detected, but only 27 com-

Phillips

Stashenko



Sacks

pounds were common to all test subjects. Phillips remarked that the most abundant volatile organic component of breath is isoprene, which he noted is a precursor to cholesterol.

Alkanes, including relatively high-molecular-weight species, constitute a large fraction of the compounds found in people's breath. "We routinely see C_4 to C_{20} compounds in normal breath samples," Phillips said. Studying the composition and concentrations of straight-chain and branched alkanes in breath "gives us an insight into the biochemistry going on in the body."

Elaborating about the basis of that insight, Phillips pointed out that in addition to normal metabolic processes that convert oxygen into water and energy, humans convert oxygen to hydroxyl and superoxide radicals and other reactive oxygen species. These potent oxidizers can react with DNA, proteins, and other biomolecules in a process referred to generically as "oxidative stress." Phillips noted that oxidative stress is known to be associated with diseases, and its by-products can serve as disease indicators.

The connection to breath testing, Phillips explained, is that the reactive oxygen species also oxidize polyunsaturated fatty acids, forming lipid-based free radicals and eventually volatile alkanes and methylated alkanes, which are excreted in breath. "That means that when we see alkanes and methylated alkanes in breath, we are actually seeing markers for oxidative stress."

To help recognize differences between sets of markers, Phillips and coworkers developed a 3-D representation of breath-component data that he referred to as a breath methylated-alkane contour. The plot presents information about each breath component's molecular structure and the relative rates at which it is produced and cleared from the body in a visual format that can be used diagnostically as a breath "fingerprint."

Basing his conclusions on statistical analysis of populations of healthy and sick test subjects, Phillips argued that breath "fingerprinting" offers a reliable, noninvasive, painless, and inexpensive way of screening patients for certain diseases. For heart transplant patients, for example, a negative breath-test result (meaning no rejection of transplanted heart) would indicate that there's no need to perform a heart biopsy, which is the standard way to test for organ rejection.

Just a few weeks ago, the Food & Drug Administration approved the breath analysis test for heart transplant rejection, Phillips announced. He noted that similar clinical studies are being conducted to develop tests for lung and breast cancer, pulmonary tuberculosis, and other conditions.

EXHALE, PLEASE University of Waterloo chemists Pawliszyn (left) and Lord develop techniques for analyzing breath samples.

Some of the other presentations in the symposium focused on sampling and sample preparation techniques as well as advances in instrumentation. For example, Heather Lord reported on a new technique in which a membrane and adsorbent trap are used in concert in an easily automated setup to extract and measure analytes for breath analysis, continuous air monitoring, and other applications. Lord serves as a laboratory manager in the research group of Janusz Pawliszyn, a professor of chemistry at the University of Waterloo, Ontario, and one of the symposium organizers.

In the Waterloo design, analytes in a carrier gas diffuse through a membrane and are trapped and concentrated on a bed of solid adsorbent. The trap is heated in a pulsed fashion at predetermined intervals to deliver the compounds to a gas chromatograph (GC) for separation and detection. Lord noted that using a nonpolar and nonporous membrane material such as polydimethylsiloxane prevents the GC from being overwhelmed by water, which is present in large concentration in breath samples.

Success with the technique depends in large part on the design of the trap and judicious choice of adsorbent and the length of trapping and heating cycles, Lord explained. For breath analysis, the group tends to use just a few milligrams of a commercial carbon molecular sieve and a flow-through trap design, she said. Another key feature is a cooling unit that includes solid-state heat pumps and heat sinks to cool the trap and increase its adsorption capacity.

Unlike some types of applications for which sampling is easily controlled, in breath analysis researchers need to account for large variations in breathing patterns and the portion of the breath being sampled (alveolar versus dead space). The Waterloo group addressed the issue by developing sampling procedures that measure internal standards such as CO_2, which is found in normal breath samples at relatively constant concentrations, Lord remarked.

Demonstrating the membrane trap method, Lord and coworkers measured methane and ethane breath levels in several test subjects. She noted that methane in breath may be an indication of incomplete absorption of carbohydrates in the small intestine. Lord reported that in addition to identifying one subject with abnormally high breath-methane levels, the study showed that the technique is easily applied to breath analysis and yields reproducible results.

An alternative approach to breath analysis was described by University of Michigan, Ann Arbor, chemistry professor Richard D. Sacks, a coorganizer of the symposium. Sacks's group has developed an instrument design and technique he refers to as "comprehensive 2-D gas chromatography." The GC method can provide very high chromatographic resolution and can be carried out with portable instruments.

In contrast with other GC systems, the Michigan setup makes use of a multisegment trap to concentrate analytes on a series of carbon adsorbent beds arranged in order of adsorption strength. Analytes are desorbed from the trap and separated in a pair of chromatography columns connected in series—a 30-meter-long column featuring a nonpolar stationary phase followed by a very short polar column.

Sacks explained that the columns are connected via a device known as a thermal modulator, which is cooled to roughly –20 °C and serves as a trap for analytes eluting from the first column. The modulator is heated rapidly every 5 seconds, thereby in-

jecting the analytes into the second column in a continuous stream of gas plugs of just millisecond duration. Separation in the short column is completed in seconds—before the next sample injection.

"In a typical 2-D analysis with a 30-minute run time, we generate hundreds of second-column separations," Sacks reported. The data are presented in a 3-D format with first- and second-column retention times plotted on the *x* and *y* axes and signal intensity on the *z* axis. Showing results of breath analyses, Sacks argued that the method offers "much greater peak capacity and one to two orders of magnitude better resolution than can be obtained with an optimized one-dimensional separation."

Instruments for 2-D gas chromatography are now available commercially, Sacks pointed out. "But they're big, heavy, and expensive, and they're tied to a huge Dewar of liquid nitrogen." The Michigan group has been miniaturizing and simplifying the apparatus. For example, they've replaced the Dewar with a smaller conventional refrigeration unit, and the GC oven is gone, thanks to special columns with built-in heating elements. "In principle, our present design is transportable," Sacks said, acknowledging that it is still fairly heavy. "We expect to simplify it even further quite soon," he remarked, adding that the portable system has performed well in breath component separations.

LOW CONCENTRATIONS of breath components can be detected selectively using a microextraction technique, developed by Pawliszyn, in which volatile analytes are trapped on a tiny fiber coated with a polymeric material. Elena E. Stashenko, a chemistry professor at Industrial University of Santander, in Bucaramanga, Colombia, reported on using the technique to analyze the compounds that remain in breath long after one has consumed certain foods or drinks or has smoked.

"You can learn a lot just by smelling your patients with the unaided nose."

More than 60 minutes after drinking cola, limonene was detected in breath samples, Stashenko reported. Similarly, thymol and eucalyptol were detected one hour after using mouthwash, and certain sulfides persisted more than one-and-a-half hours after consuming onions, she noted.

Stashenko also described derivitization methods in which various hydrazine compounds such as pentafluorophenylhydrazine are used to convert volatile carbonyl species in breath to hydrazones. The technique provides selective and sensitive detection of breath analytes and can even be used to separate enantiomers, she said.

With continuing advances in analytical techniques and instrumentation, breath analysis is poised to move from scientific research topic to medical diagnostic tool. As Menssana's Phillips speculated, "Physicians and patients of the 21st century may eventually come to think of a breath test in much the same way as we now think of a chest X-ray or a blood test: as an inexpensive and convenient screening test that can detect several diseases in their earliest and most treatable stages." ■